Reference: *Biol. Bull.* **208:** 145–155. (April 2005)

Characterization of Symbiont Populations in Life-History Stages of Mussels From Chemosynthetic Environments

JENNIFER L. SALERNO[1], STEPHEN A. MACKO[2], STEVE J. HALLAM[3], MONIKA BRIGHT[4], YONG-JIN WON[5], ZOE McKINESS[6], AND CINDY L. VAN DOVER[1,*]

[1] *Biology Department, The College of William & Mary, Williamsburg, Virginia 23187;* [2] *Department of Environmental Sciences, University of Virginia, Charlottesville, Virginia 2290;* [3] *Monterey Bay Aquarium Research Institute, 7700 Sandholdt Road, Moss Landing, California 95039;* [4] *Department of Marine Biology, Institute of Ecology and Conservation Biology, University of Vienna, Althantstrasse 14, 1090 Vienna, Austria;* [5] *Dept. of Life Sciences, Ewha Womans University, Science C Building, 11-1 Daehyon-Dong, Sodaemun-Ku, Seoul, 120–750, Korea; and* [6] *Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138*

Abstract. The densities of chemoautotrophic and methanotrophic symbiont morphotypes were determined in life-history stages (post-larvae, juveniles, adults) of two species of mussels (*Bathymodiolus azoricus* and *B. heckerae*) from deep-sea chemosynthetic environments (the Lucky Strike hydrothermal vent and the Blake Ridge cold seep) in the Atlantic Ocean. Both symbiont morphotypes were observed in all specimens and in the same relative proportions, regardless of life-history stage. The relative abundance of symbiont morphotypes, determined by transmission electron microscopy, was different in the two species: chemoautotrophs were dominant (13:1–18:1) in *B. azoricus* from the vent site; methanotrophs were dominant (2:1–3:1) in *B. heckerae* from the seep site. The ratio of CH_4:H_2S is proposed as a determinant of the relative abundance of symbiont types: where CH_4:H_2S is less than 1, as at the Lucky Strike site, chemoautotrophic symbionts dominate; where CH_4:H_2S is greater than 2, as at the seep site, methanotrophs dominate. Organic carbon and nitrogen isotopic compositions of *B. azoricus* ($\delta^{13}C = -30‰$; $\delta^{15}N = -9‰$) and *B. heckerae* ($\delta^{13}C = -56‰$; $\delta^{15}N = -2‰$) varied little among life-history stages and provided no record of a larval diet of photosynthetically derived organic material in the post-larval and juvenile stages.

Received 17 December 2003; accepted 9 February 2005.
* To whom correspondence should be addressed. E-mail: clvand@wm.edu

Introduction

Mussels in the genus *Bathymodiolus* are biomass dominants at many known deep-sea hydrothermal vent and cold seep habitats, where they are host to endosymbiotic, autotrophic bacteria in their gills. Anatomical and nutritional relationships between the symbionts and their adult hosts is well documented (*e.g.*, Distel *et al.*, 1995; Robinson *et al.*, 1998; Southward *et al.*, 2001; Fiala-Médioni *et al.*, 2002; Raulfs *et al.*, 2004). Although bathymodiolin mussels may be able to obtain some nutrition by suspension feeding (Le Pennec *et al.*, 1990; Page *et al.*, 1991; Fujiwara *et al.*, 1998), most of the nutrition of adult mussels is derived from their symbionts (Fisher *et al.*, 1988; reviewed in Fisher, 1990; Childress and Fisher, 1992; Kochevar *et al.*, 1992). Larval stages of vent mussels are pelagic and have been described as planktotrophic (Lutz *et al.*, 1980; Berg, 1985; LePennec and Beninger, 2000), but the distribution of mussel larvae in the water column, the nature of their planktonic diet, and the trophic transition that takes place as they become benthic are all unknown.

Some mussel species host only chemoautotrophic (also referred to as thiotrophic), sulfur-oxidizing bacterial symbionts (Nelson *et al.*, 1995; Fujiwara *et al.*, 2000); other species host only methanotrophic symbionts (Fujiwara *et al.*, 2000; Barry *et al.*, 2002); and still others host both types of bacteria ("dual symbionts"; Fisher *et al.*, 1993; Robinson *et al.*, 1998; Fiala-Médioni *et al.*, 2002). Dual symbionts

provide obvious advantages to host individuals recruiting to environments where the availability of substrates is unpredictable or fluctuating (Cavanaugh *et al.*, 1992; Robinson *et al.*, 1998; Fiala-Médioni *et al.*, 2002), but the host is also challenged to recognize and sequester two different microbial types within its cells, while excluding pathogenic or otherwise harmful bacteria. There is some empirical evidence that the relative abundance of dual symbionts within adult mussels of a given species can vary in response to environmental parameters (Trask and Van Dover, 1999; Fiala-Médioni *et al.*, 2002), although *in situ* studies involving transplant experiments have yet to be reported, and the manner through which these symbiont populations are regulated is unclear. Although the mode or modes of acquisition of dual symbionts in the earliest life-history stages of mussels is also uncertain, a recent report (Won *et al.*, 2003) provides evidence for environmental acquisition of chemoautotrophic symbionts in *Bathymodiolus azoricus* von Cosel *et al.*, 1999; and *B. puteoserpentis* von Cosel *et al.* 1994 from hydrothermal vents. If symbionts are acquired from the environment, rather than by maternal transfer, then acquisition of dual symbionts need not be simultaneous and may be a response to ontogenetic or environmental conditions of the host mussels. Furthermore, it is not inconceivable that juveniles and adults might use sulfide and methane in different proportions so as to minimize intraspecific competition, or that juveniles and adults occupy different microzones within diffuse flow vents with different relative availabilities of sulfide and methane, and that these conditions might be reflected in the relative abundance of the symbiont types (Trask and Van Dover, 1999; Colaço *et al.*, 2002; Fiala-Médioni *et al.*, 2002).

Adults of *B. azoricus* from the Lucky Strike vent site on the Mid-Atlantic Ridge (Fig. 1) are known to host chemoautotrophic and methanotrophic symbionts in their gills; the evidence is based on ultrastructural, biochemical, isotopic, and immunological characterization (Fiala-Médioni *et al.*, 2002). Adults of *B. heckerae* from the Blake Ridge seep site off the coast of South Carolina (Fig. 1) are also reported to host dual symbionts; this report is based on ultrastructural and isotopic evidence (Van Dover *et al.*, 2003). In this study, we used transmission electron microscopy (TEM) to explore the composition and density of symbionts in early life-history stages (*i.e.*, post-larvae and juveniles) of these species, as well as in adults. Mussel recruits often occur in large numbers (>1000 m^{-2}) in mussel beds (CLVD, pers. obs.). The most recent arrivals (post-larvae) are between 0.6 and 1.2 mm in shell length and are recognizable by the presence of prodissoconch I and II shells and the lack of dissoconch shell growth. In addition to lines of demarcation, prodissoconch and dissoconch shells are conveniently differentiated by shell color: prodissoconch shells are pink or red; dissoconch shell material is yellow.

We aimed in this study to determine whether chemoautotrophic and methanotrophic symbionts are present in the very earliest benthic stages, which would indicate simultaneous or near-simultaneous acquisition of both symbiont types early in the life history of the mussels. We also asked whether the relative abundance of chemoautotrophs and methanotrophs includes an ontogenetic factor. In addition to using transmission electron microscopy for quantitative assessment of symbiont populations, we used carbon and nitrogen stable isotopes in an attempt to detect isotopic remnants of a photosynthetically based larval diet in post-larval tissues, to interpret the relative abundance of symbiont types within and between mussel species, and to determine whether the contribution of chemoautotrophic and methanotrophic production to host nutrition varied with the size of the mussel.

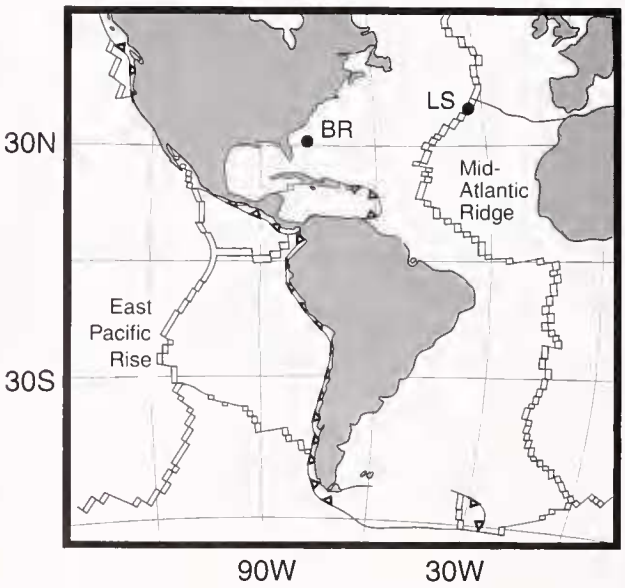


Figure 1. Location of sampling sites. LS = Lucky Strike hydrothermal vent, Mid-Atlantic Ridge; BR = Blake Ridge cold seep.

Materials and Methods

Sample collection

The deep-submersible vehicle *Alvin* was used to collect samples of adult mussels and their associated fauna. *Bathymodiolus azoricus* mussels were collected in July 2001 from Eiffel Tower at the Lucky Strike hydrothermal vent field [Mid-Atlantic Ridge, 37°17.5′N, 32°16.5′W; 1687 m; see Van Dover *et al.* (1996) for site description]. *Bathymodiolus heckerae* was collected in September 2001 from the Blake Ridge methane-hydrate seep [western Atlantic, 32°31′N, 76°12′W; 2170 m; see Van Dover *et al.* (2003) for site description]. The mussels were rinsed over a 250-μm sieve with chilled (4 °C), 10-μm filtered seawater. To collect

post-larval and juvenile mussels, fresh, sieved material was sorted under a dissecting microscope.

Mussels were separated into classes based on shell color, shell morphology, and shell length. The smallest size class consisted of post-larval mussels with pinkish-red prodissoconchs (I and II) and with shell lengths within the range of 0.12 to 0.60 mm. Early juveniles, herein referred to as "J1", were characterized by the presence of a narrow band of yellow dissoconch shell and by shell lengths within the range of 0.6 to 1.2 mm. Four additional juvenile size classes were designated, with shell lengths in the following ranges: J2: 1.2–2.4 mm; J3: 3.6–4.8 mm; J4: 4.8–6.0 mm; and J5: 6.0–8.4 mm.

Transmission electron microscopy

A 0.5-cm-wide section of tissue was dissected from the middle of the gill of adult and larger juvenile specimens. Where mussels were too small to be dissected (*i.e.*, post-larvae and juvenile stages J-1 and J-2), the shells were cracked to allow fixative to penetrate the tissue. *Bathymodiolus azoricus* tissues were fixed for about 3 weeks in 3% glutaraldehyde with 0.1 *M* cacodylate buffer and 0.4 *M* NaCl (pH 7.8). *Bathymodiolus heckerae* tissues were fixed for about 48 hours in 3% glutaraldehyde with 0.1 *M* phosphate buffer and 0.25 *M* sucrose (pH 7.4). Samples were rinsed in 0.1 *M* phosphate buffer containing 0.25 *M* sucrose and were post-fixed in a 1% osmium tetroxide solution. The shells of post-larvae and juveniles were dissolved by a 24-h immersion in 2.5 g EDTA/100 ml buffer solution. Specimens were dehydrated in a graded acetone series and stained *en bloc* with 2% uranyl acetate. Gill tissue or whole individuals were infiltrated with Embed 812 epoxy embedding medium, polymerized, cut into 70-nm to 80-nm thin sections, and stained with lead citrate. Gill sections were viewed with a Zeiss 109 transmission electron microscope.

Densities of bacterial morphotypes

The density of bacterial morphotypes is defined here as the number of methanotrophic or chemoautotrophic cells per 15.5 μm^2 within the apical region of bacteriocytes in transverse section (Fig. 2). The areal dimension and location were chosen because they approximately cover the region of a bacteriocyte that contains symbionts. For each specimen, three transverse sections were cut through the middle of the gill filaments (3 to 6 gill filaments per section) at intervals greater than the maximum diameter of the bacteria (*i.e.*, > 2.0 μm) to ensure that a particular bacterial cell was counted only once. Fifteen 15.5-μm^2 areas per section were haphazardly selected at low magnification (240×) for bacterial cell counts. Where an area selected haphazardly at low magnification was not located in the apical region of a bacteriocyte, the specimen was adjusted until the apical region of that bacteriocyte filled the field of view. Counts were carried

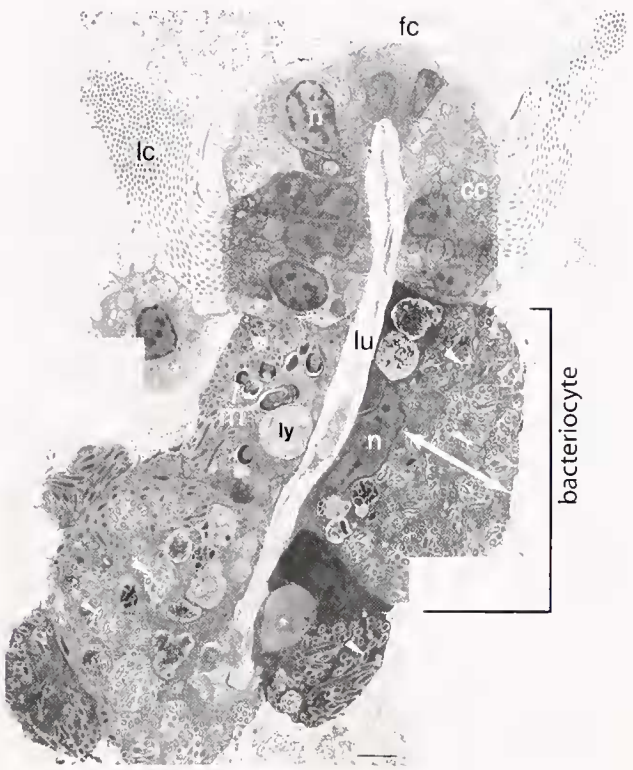


Figure 2. Transmission electron micrograph mosaic of a transverse section through a post-larval specimen of *Bathymodiolus azoricus* (0.12 to 0.60 mm in shell length) gill filament. cc, ciliary cell; fc, frontal cilia; lc, lateral cilia; lu, lumen; ly, lysosomal-like residual body; n, nucleus; m, methanotrophic morphotypes; arrowheads, chemoautotrophic morphotypes; double-ended arrow, apical region of bacteriocyte. Scale bar = 2 μm.

out at high magnification (48,000×) and included all bacterial cells that were in the field of view and those cells that were intersected by the upper and right margins.

The density of bacterial morphotypes was estimated for mussels of different sizes within each species, as follows: *Bathymodiolus azoricus*—3 post-larvae, 5 J1, 5 J2, 5 J3, and 4 adults; *B. heckerae*—3 post-larvae, 5 J1, 4 J2, 5 J3, and 4 adults. The Kruskal-Wallis nonparametric test for a one-way design was used to assess variations in median density of each morphotype within a mussel species ($\alpha = 0.05$).

Stable isotope analyses

Foot, gill, and mantle tissue was dissected, variously, from adult mussels (>20 mm in shell length) and placed in a drying oven (70 °C) for 24 h. Due to their small size, post-larval and juvenile mussels (<8 mm in shell length) were pooled [post-larvae: 35 individuals per pooled sample; juvenile size classes (J1 through J5): 2 individuals per pooled sample]. These pooled samples were dried whole, then soaked in 10% HCl to remove the shell carbonate, and then re-dried. Subsamples of dried, acidified tissues were

ground to a fine powder and packaged in tin capsules. Samples were transformed to CO_2 and N_2 for isotope analysis with a Carlo Erba elemental analyzer coupled to an OPTIMA stable isotope ratio mass spectrometer (Micromass, Manchester, UK). Carbon and nitrogen isotopes were determined with a single combustion in a dual-furnace system composed of an oxidation furnace at 1020 °C and a reduction furnace at 650 °C. The resulting gases were purified by gas chromatography, chemically dried, and injected into the source of the mass spectrometer by continuous flow.

Stable isotope ratios are reported in the following notation: $\delta^X E = [R_{sample}/R_{standard} - 1]\ 10^3$ (‰), where X is the heavy isotope of element E, and R is the abundance ratio of the heavy to light isotopes of that element (*i.e.*, $^{13}C/^{12}C$ and $^{15}N/^{14}N$). Stable isotope compositions were calculated relative to the international standards for carbon (Pee Dee Belemnite limestone, PDB) and nitrogen (atmospheric N_2, air), which have defined $\delta^X E$ values of 0.0‰. Isotope values are reported as means (± standard deviation). Statistical comparisons of isotopic compositions between small and large mussels were made after defining a *post hoc* 8-mm boundary based on the relationship between isotopic composition and shell length.

Results

Gill ultrastructure

The number of gill filaments and the length of the dorsal-ventral axis of the gill filaments increase with shell length and volume of the individual. The increase in gill-filament length reflects an increase in the number of bacteriocytes and intercalary cells constituting the gill filament. Cells exhibiting two distinct morphotypes were observed with transmission electron microscopy within gill epithelial cells (bacteriocytes) of all stages of *Bathymodiolus azoricus* and *B. heckerae* (total number of specimens examined = 43) and were interpreted to be bacteria (Figs. 2, 3). Both bacterial morphotypes, either singly or in groups of two or more cells, were contained in vacuoles surrounded by a peribacterial membrane. The larger, round-to-oval-shaped morphotypes did not differ in size within the different host mussel species, and had an overall mean diameter of 1.1 μm (± 0.06 SE; n = 165). These large cells contained stacks of complex intracytoplasmic membranes, characteristic of type I or type X methanotrophic bacteria (Cavanaugh *et al.*, 1992). The smaller morphotype was also similar in size within the different host mussel species, and had an overall mean diameter of 0.31 μm (± 0.05 SE; n = 165). This smaller cell type was coccid or, less frequently, rod-shaped, lacked intracellular membranes, and resembled chemoautotrophic bacteria (Cavanaugh *et al.*, 1992). Because these two morphotypes were of the same general size and ultrastructure as the two types of symbionts described from other bathymodiolin mussels (Cavanaugh *et al.*, 1987; Fisher *et al.*, 1993; Distel *et al.*, 1995; Fiala-Médioni *et al.*, 2002), we refer to them herein as chemoautotrophic and methanotrophic symbiont morphotypes, or simply as chemoautotrophs and methanotrophs.

Divisional stages of chemoautotrophic and methanotrophic bacterial morphotypes were observed in all size classes of both mussel species. Transverse sections of bacteriocytes that contained only a single symbiont type were rarely observed. Apparent lysosomal digestion of symbionts (Fiala-Médioni *et al.*, 1986, 2002; Kochevar *et al.*, 1992) was indicated by the presence of cellular components resembling lysosomal residual bodies in the basal portion of the bacteriocytes (Figs. 2, 3). These bodies appeared to contain remnants of partially digested bacterial cells of both types and were observed regularly in both species of mussel and in all size classes examined.

Possible instances of endo- or exocytosis of symbionts (*e.g.*, Fig. 4A) were observed in several *B. azoricus* and *B. heckerae* individuals, but, due to the static nature of transmission electron microscopy, we could neither infer which of these two processes was taking place nor even eliminate the possibility that this observation was an artifact of fixation. Abnormally large vacuoles filled with chemoautotrophic or methanotrophic bacterial morphotypes, or both, were occasionally observed in *B. azoricus* mussels (Fig. 4B).

In several *B. azoricus* individuals, bacteriocytes seemed to be separated from adjacent bacteriocytes and were rounded off at their basal portion (Fig. 4C). In other cases, bacteriocyte cell membranes and most of the peribacterial membranes were disrupted (Fig. 4D).

Chemoautotrophic and methanotrophic bacterial morphotypes were also observed in the mantle epithelium of post-larval and juvenile *B. azoricus* and *B. heckerae* mussels (Fig. 4E, F). Mantle epithelial cells containing symbiont morphotypes were similar in ultrastructure to gill bacteriocytes. Cellular components resembling lysosomal residual bodies were also observed near the basal nuclei of mantle bacteriocytes. Bacteria-like bodies, with dimensions and ultrastructural characteristics resembling those of chemoautotrophic and methanotrophic symbiont morphotypes of the gill and mantle, were also observed in the gut lumen of several post-larvae.

Density of bacterial symbiont morphotypes

Total average density of symbionts was 4 to 5 times greater in *B. azoricus* than in *B. heckerae* (Fig. 5). Methanotrophic morphotype densities in *B. azoricus* were uniformly low (mean density < 4 cells per 15.5 μm^2) in all size classes examined; chemoautotrophic morphotype densities (mean density between 23 and 40 cells per 15.5 μm^2 in 17 of 22 specimens) were an order of magnitude greater than

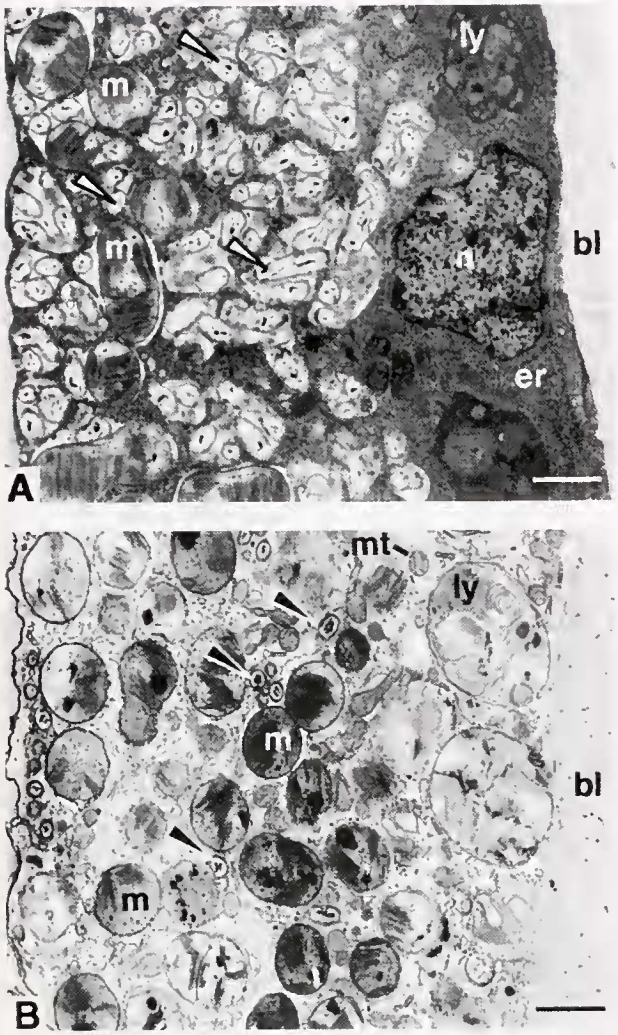


Figure 3. Transmission electron micrographs of transverse sections through (A) *Bathymodiolus azoricus* and (B) *B. heckerae* mussel gill filaments of juveniles (J1 size class). bl, basal lamina; er, endoplasmic reticulum; ly, lysosomal-like residual body; mt, mitochondrion; n, nucleus; m, methanotrophic morphotypes; arrowheads, chemoautotrophic morphotypes. Scale bars = 1 μm.

methanotrophic morphotype densities (Fig. 5A). Variation in densities of both morphotypes was maximal in the smallest, post-larval size class. No significant differences in median symbiont densities were detected in Kruskal-Wallis comparisons of size-class pairs within *B. azoricus* (chemoautotrophs, $P = 0.115$; methanotrophs, $P = 0.383$).

Methanotrophic symbionts accounted for more than 60% of the total bacterial symbiont density in *B. heckerae*, compared to less than 10% in *B. azoricus* (Fig. 5). Methanotrophic and chemoautotrophic densities were uniformly low in *B. heckerae* (mean density of both morphotypes combined, < 15 cells per 15.5 μm^2) in all size classes examined (Fig. 5B). The median density of symbionts in *B. heckerae* did not differ among the size-classes examined (chemoautotrophs, $P = 0.67$; methanotrophs, $P = 0.342$).

Stable isotopic compositions

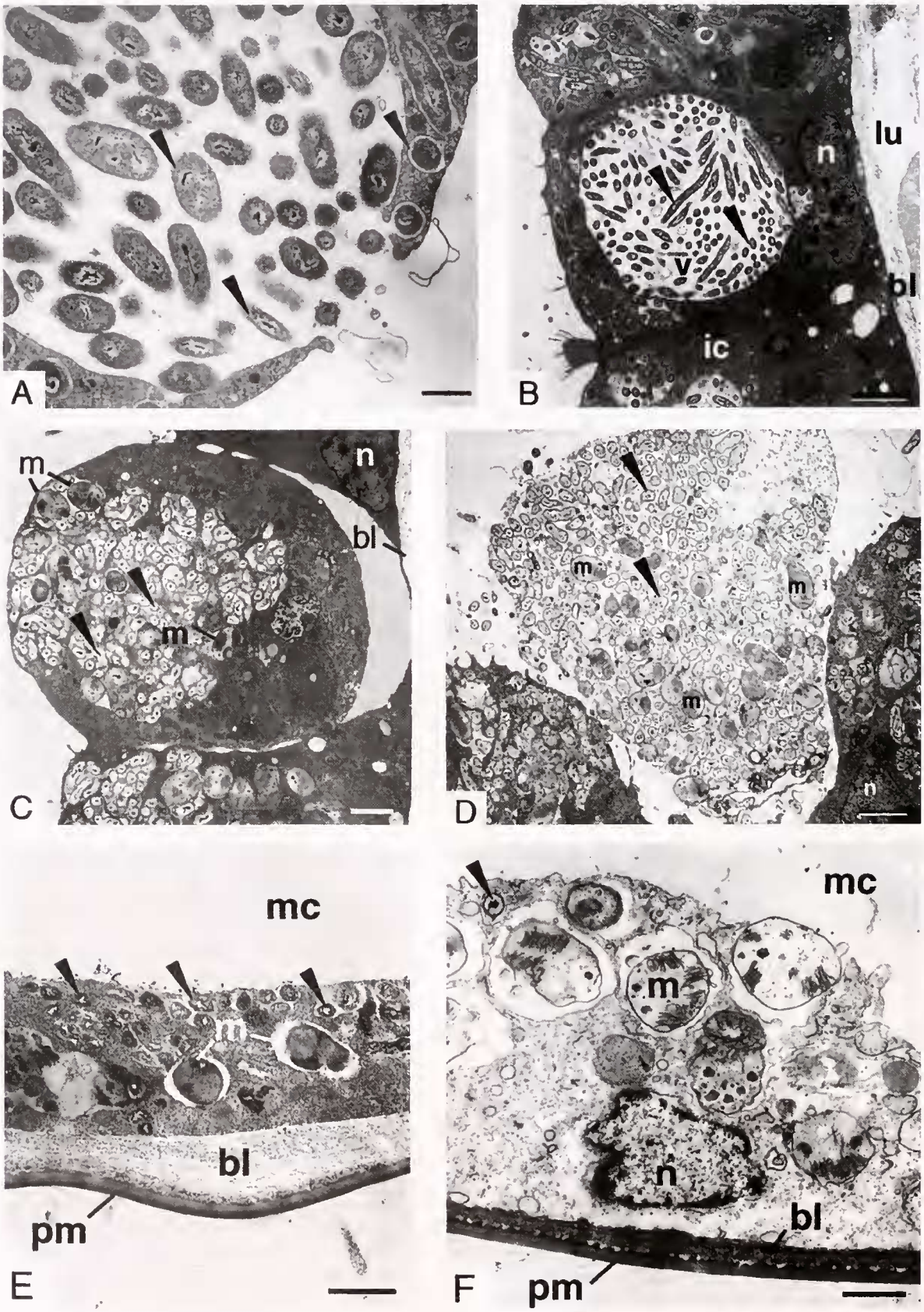
A difference of more than 25‰ was measured in the $\delta^{13}C$ compositions (Fig. 6) of *B. azoricus* (~ -30‰) and *B. heckerae* (~ -56‰). Within *B. azoricus*, the $\delta^{13}C$ composition was enriched in ^{13}C (by 3‰) in juvenile mussels (<8 mm shell length) compared with larger mussels (>20 mm shell length). Average $\delta^{15}N$ values of mussel tissues (Fig. 6) were also different in the two species (*B. azoricus:* ~ -9‰; *B. heckerae:* ~ -2‰), and there was a small (~1.6‰) difference in the nitrogen isotopic composition of small and large individuals of *B. azoricus*. Average $\delta^{15}N$ values were enriched in ^{15}N by ~5.5‰ in large individuals of *B. heckerae* (>20 mm) compared to small ones (<8 mm), whether the comparison was made using values for gill or mantle in the larger individuals.

Discussion

Descriptive characteristics of the symbionts

Gill tissues of *Bathymodiolus azoricus* and *B. heckerae* post-larvae from vent and seep mussel beds contain cells that we infer to be methanotrophic and chemoautotrophic endosymbionts on the basis of their size and ultrastructural characters. The presence of both symbiont morphotypes in the earliest, post-larval benthic stage of the mussels suggests that the mussels are autotrophically competent to oxidize sulfide and methane as soon as they arrive at a vent site or very shortly thereafter. The absence of symbionts in post-larvae would have been consistent with a horizontal mode of transmission; the presence of symbionts in post-larvae does not allow us to resolve the mode of symbiont transmission.

The presence of chemoautotrophic and methanotrophic symbiont types was not limited to gill tissues; symbionts were also found in mantle epithelial cells of post-larvae and juveniles. Bacteriocytes located along that surface of the mantle exposed to the mantle cavity, and thus to the water flow generated by gill cilia in post-metamorphic mussels, were morphologically indistinguishable from bacteriocytes in the gill epithelium. Qualitative observations indicate that bacteriocytes in both locations contain similar densities and ratios of endosymbionts. Mantle and gill bacteriocytes also contained cellular components resembling lysosomal residual bodies, consistent with digestion of bacteria and nutritional reliance on symbionts by host mussels (Fisher, 1990; Cavanaugh *et al.*, 1992; Fiala-Médioni *et al.*, 2002; Barry *et al.*, 2002). The observation of mantle bacteriocytes is not unprecedented, although this is, to our knowledge, the first record for a vent mussel. Streams *et al.* (1997) observed coccid bacteria resembling gill endosymbionts in the mantle


A
B
lu
n
v
bl
ic
C
m
n
bl
m
D
m
m
m
n
E
mc
m
bl
pm
F
mc
m
n
bl
pm

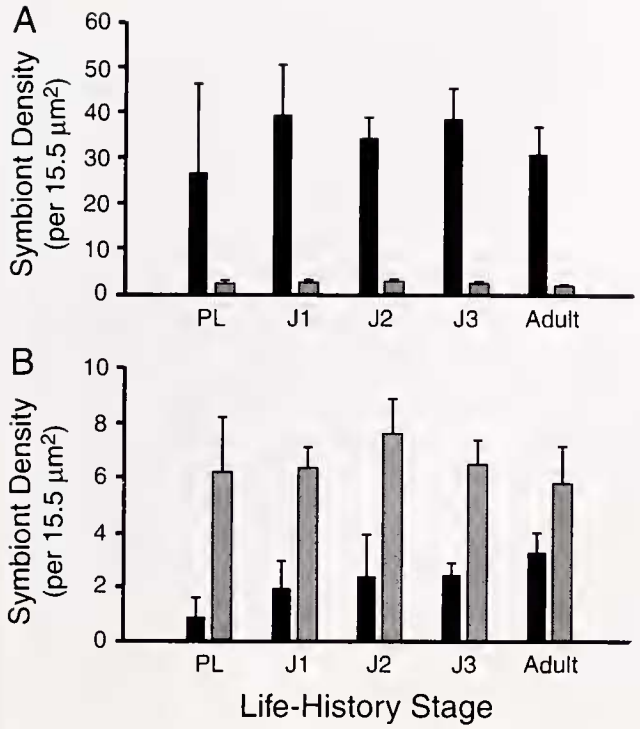


Figure 5. Symbiont densities per 15.5 μm^2 ($\pm$ SD) in gill tissues. (A) *Bathymodiolus azoricus.* (B) *B. heckerae.* PL = post-larvae; J1–J3 = juvenile size classes 1–3 (see text). Black bars, chemoautotrophic morphotype; grey bars, methanotrophic morphotypes.

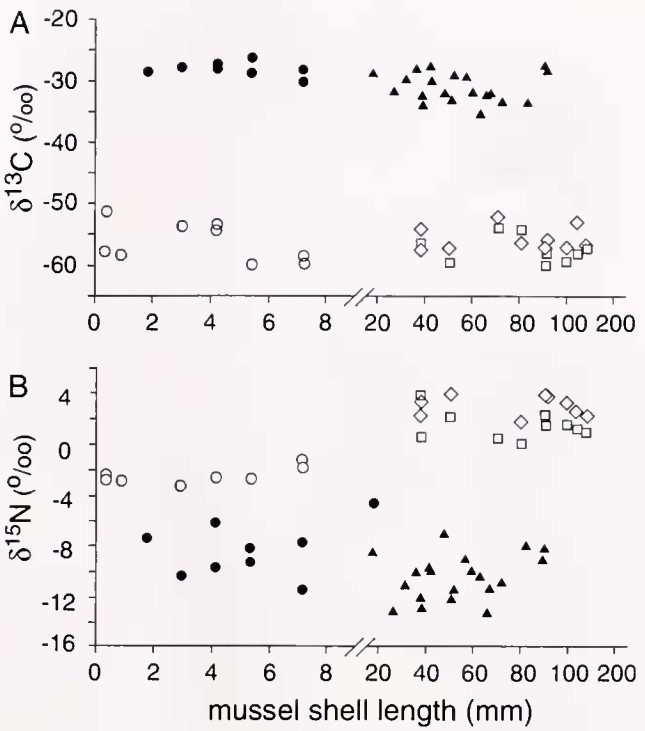


Figure 6. *Bathymodiolus* spp. stable isotopic composition of tissues. (A) $\delta^{13}C$ (‰) *vs.* shell length; (B) $\delta^{15}N$ (‰) *vs.* shell length. *B. azoricus:* filled symbols; *B. heckerae:* open symbols. For whole mussels < 8 mm (○ or ●), whole animals (acid-treated to dissolve shells) were pooled and analyzed (see Materials and Methods). For mussels > 20 mm, ▲ = foot tissue; ◇ = gill tissue; □ = mantle tissue.

and foot tissue of juvenile (shell length = 4 to 8 mm) seep mussels (*Bathymodiolus childressi*) from the Gulf of Mexico. All symbionts exhibited morphotypes of type I methane-oxidizing bacteria and actively incorporated carbon from methane during ^{14}C tissue autoradiography experiments. Given the origins of gill primordia as epithelial protuberances of the mantle at the base of the foot during larval development in some bivalve species (Chaparro *et al.,* 2001), mantle cells, rather than gill cells, may be the locus of initial infection of environmentally acquired symbionts (Streams *et al.,* 1997). Furthermore, the volume of gill bacteriocytes is limited in smaller individuals. Mantle symbionts may provide an important additional source of nutrition to post-larval and juvenile mussels.

In addition to possible lysosomal digestion of gill and mantle symbionts, we occasionally observed gill and mantle bacteriocytes associated with large clusters of extracellular symbionts in both mussel species, but we could not tell whether the host cells were engaged in endocytosis or exocytosis at the time of fixation (but see also Won *et al.,* 2003, wherein endocytosis in a bathymodiolin species is inferred from ultrastructural studies).

Bacteriocytes in apparent stages of degradation were occasionally observed, although we cannot discount the possibility that the degraded cells are artifacts of the fixation process. In some cases, degrading cells are round and separate laterally from adjacent bacteriocytes, but host cytoplasm and organelles and symbionts remain intact. In other degrading cells, host cell membranes and peribacterial membranes are disrupted, host cytoplasm and organelles are lost, and symbionts appear to be released from the host cell. If these "abnormal" bacteriocytes indeed represent a degradation sequence, the intact nature of the symbionts stands in

Figure 4. (A) Example of a breach in a gill bacteriocyte membrane, indicative of endo- or exocytosis; scale bar = 2.0 μm. (B) Large vacuole filled with chemoautotrophic morphotypes in a gill bacteriocyte; scale bar = 1.0 μm. (C) Gill bacteriocyte separating from surrounding bacteriocytes; scale bar = 1.0 μm. (D) Bacteria expelled from a gill bacteriocytes; scale bar = 0.5 μm. (E, F) Mantle tissue of post-larval specimens of *Bathymodiolus azoricus* and *B. heckerae,* respectively; scale bar (E) = 1.0 μm, scale bar (F) = 1.5 μm. bl, basal lamina; ic, intercalary cell; lu, lumen; mc, mantle cavity; n, nucleus; v, vacuole; m, methanotrophic morphotypes; pm, protein matrix; arrowheads, chemoautotrophic morphotypes.

contrast to digested symbionts observed during the process of cell death in *Riftia pachyptila* (Bright and Sorgo, 2003). The specific processes of cell death, as well as the fate of the symbiotic bacteria, remain to be studied in bathymodiolin mussels.

Quantitative comparisons of symbiont densities

Symbiont population densities are consistently different in the host species studied. Total (*i.e.*, chemoautotrophic and methanotrophic) symbiont density within gill bacteriocytes was 4 to 5 times greater in *B. azoricus* than in *B. heckerae* and was independent of size class. The ratio of chemoautotrophs to methanotrophs was also species-specific and independent of size class, with chemoautotrophs much more numerous than methanotrophs (13:1 to 18:1) in *B. azoricus*, and with methanotrophs nearly equal to, or moderately more numerous than, chemoautotrophs (2:1 to 3:1) in *B. heckerae*. Although the total symbiont densities are different in the two species, the volume of a gill bacteriocyte occupied by symbionts is qualitatively similar, because the two symbiont morphotypes differ in their size and relative abundance.

The lack of significant differences in symbiont densities and relative abundances among the size classes examined within the two mussel species suggests that there is no niche separation at the level of symbiont populations between the juvenile mussels and the adults. Density data also offer no evidence for an acquisition priority for one symbiont type over the other in either mussel species, or for a systematic ontogenetic shift in the relative density of symbiont morphotypes. We infer that gill symbionts of post-larvae, juveniles, and adults within a mussel population compete for methane and sulfide in the same relative proportions, and that the symbiont densities are regulated. The mode of that regulation—whether by host control mechanisms, by density-dependent control mechanisms elicited by the bacterial symbionts, or by the environment—remains unknown.

Relationships among symbiont densities, stable isotope compositions, life-history stages, and the environment

The dominance of chemoautotrophs over methanotrophs in *B. azoricus*, and of methanotrophs over chemoautotrophs in *B. heckerae*, is consistent with the limited data available on sulfide and methane availability at the Lucky Strike vent and the Blake Ridge seep. At the Eiffel Tower site (Lucky Strike), sulfide concentrations (2.1 m*M*) were higher than methane concentrations (0.68 m*M*; Charlou *et al.*, 2000) in end-member fluids (*i.e.*, high-temperature fluids [350 °C] emanating from black smokers). At the methane-hydrate seep site, pore-water methane concentrations were as much as 3.4 m*M*, while dissolved sulfide concentrations in pore-waters were 1.3 m*M* (Paull *et al.*, 1996). The only estimates available thus suggest that the ratio of CH_4:H_2S is less than 1 at the Lucky Strike site, where chemoautotrophic symbionts dominate, and greater than 2 at the seep site, where methanotrophs dominate. While the relative dominance of chemoautotrophs and methanotrophs can be site-specific within a species, presumably in response to environmental availabilities of sulfide and methane (Trask and Van Dover, 1999; Fiala-Médioni *et al.*, 2002), coordinated measurements of symbiont density and the chemical environment of the host mussels have yet to be undertaken.

Stable carbon and nitrogen isotopic compositions of mussel tissues were also species-specific. The 25‰ difference in $\delta^{13}C$ between the tissues of seep mussels and vent mussels reflects differences in the source of methane at the seep (bacterially derived; $\delta^{13}C_{methane} = -67.8‰$; Paull *et al.*, 1995, 2000) and at the vent (thermogenically derived; $\delta^{13}C_{methane} = -13.7‰$ to $-12.7‰$; Radford-Knoery *et al.*, 1998; Charlou *et al.*, 2002), as well as the relative contributions of methanotrophs and chemoautotrophs to mussel nutrition. The range of $\delta^{13}C$ values reported here for *B. azoricus* adult and juvenile mussels collected from Eiffel Tower in 2001 ($-26.3‰$ to $-35.6‰$) is very close to the range of values reported for adult mussels ($-28.7‰$ to $-33.4‰$) collected from Eiffel Tower in 1996 (Trask and Van Dover, 1999) and is within the range reported for bathymodiolin mussels that host only chemoautotrophic endosymbionts (Fisher, 1990). The methanotroph-to-chemoautotroph ratio for *B. azoricus* from Eiffel Tower at Lucky Strike (this study) is identical to that of *B. azoricus* adults sampled from the same locale in 1996 (Trask and Van Dover, 1999). Because carbon stable-isotope ratios in mussel tissues and ratios of methanotrophs to chemoautotrophs in *B. azoricus* remained essentially unchanged between sampling periods, and assuming that ratios of methanotrophs to chemoautotrophs shift in response to fluid chemistry, it seems likely that the relative availability of methane and sulfide at Eiffel Tower was the same in 1996 and 2001.

The relationships among relative symbiont density, relative availability of CH_4 and dissolved H_2S, and carbon stable-isotopic compositions may prove to be relatively straightforward in mussels with dual symbionts on the Mid-Atlantic Ridge, and independent of the mussel species (Trask and Van Dover, 1999; Colaço *et al.*, 2002; Fiala-Médioni *et al.*, 2002). The carbon isotopic composition of the source methane varies from site to site on the Mid-Atlantic Ridge, but falls in a range of roughly $-13‰$ to $-18‰$ (Charlou *et al.*, 2002), and cannot explain much of the observed variation in the carbon isotopic composition of mussel gills at these vent sites. Interpretation of carbon isotopic fractionation associated with dual symbioses in mussels may be complicated by the availability of methane-derived CO_2 as a substrate for sulfide-based autotrophic metabolism within the bacteriocytes (Fisher, 1993; Fiala-Médioni *et al.*, 2002). Nevertheless, $\delta^{13}C$ values reported

for *B. azoricus* and *B. puteoserpentis* from Mid-Atlantic Ridge vents are positively related to the ratio of CH_4 to H_2S in the end-member fluids (Fig. 7). Ratios of methanotrophs to chemoautotrophs are so far reported only for *B. azoricus* from Lucky Strike and Menez Gwen (Trask and Van Dover, 1999; Fiala-Médioni *et al.*, 2002; this study) and for *B. heckerae* from the Blake Ridge seep (this study), and are consistent with the model of increasing density of methanotrophs as the ratio of CH_4 to H_2S increases (Fig. 7). The relationships in Figure 7 are intriguing, but low-temperature fluid chemistry, symbiont densities, and isotopic compositions of fluids and host mussel tissues remain to be determined in a systematic manner across a range of CH_4:H_2S values for any given species. These relationships should ultimately prove powerful in the analysis of energy flow in dual-symbiont mussel systems.

Tissues from *B. heckerae* were consistently more enriched in ^{15}N than tissues from *B. azoricus*. Lacking any information about the isotopic composition of the nitrogen source used by these symbioses at either the seep or the vent site, we cannot interpret these differences. Within *B. heckerae*, larger mussels (> 40 mm in shell length) had nitrogen isotopic compositions about 5.5‰ more enriched in ^{15}N than post-larval and juvenile mussels (Fig. 6), consistent with an increased contribution of photosynthetically derived organic material in larger mussels. Nevertheless, alternative explanations, including variation in the isotopic composition of the nitrogen source available to the different-sized mussels, seem just as likely.

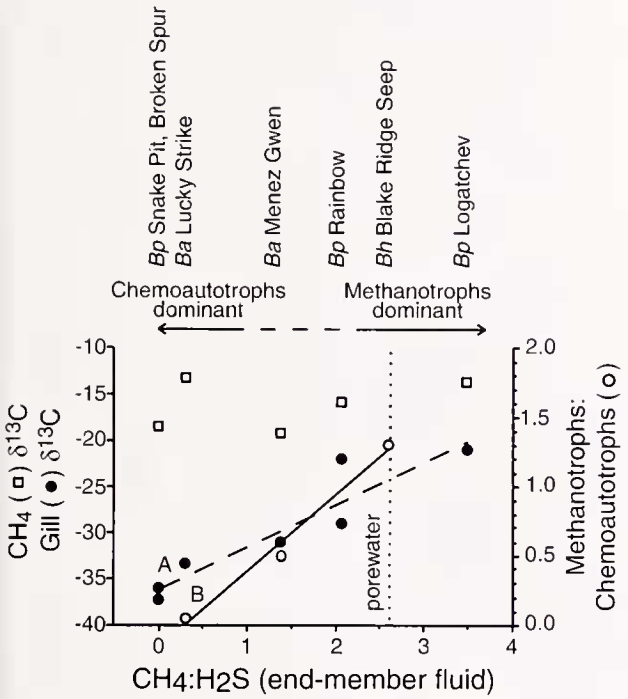


Figure 7. Relationship between the $\delta^{13}C$ of source methane, the $\delta^{13}C$ of mussel gill tissue, the ratio of methanotrophs to chemoautotrophs in gill bacteriocytes of mussels (identified as *Bp*: *Bathymodiolus puteoserpentis*; *Ba* = *B. azoricus*; *Bh* = *B. heckerae*), and the ratio of methane to sulfide (CH_4:H_2S) in end-member fluids of vent sites. CH_4:H_2S for seep porewater is indicated by the dotted vertical line. Sites are identified at the top of the figure. Line A: $\delta^{13}C_{gill}$ = 4.5 (CH_4:H_2S)—36‰; r^2 = 0.87. Line B: Ratio of methanotrophs to chemoautotrophs = 0.55 (CH_4:H_2S)—0.17‰; r^2 = 0.98. CH_4:H_2S regimes where chemoautotrophs or methanotrophs are predicted to dominate in mussels with dual symbionts are identified by the horizontal arrows; the dashed line connecting the arrows emphasizes the fact that this is a model. Methane isotope data and CH_4:H_2S ratios are from Charlou *et al.* (2002) and Paul *et al.* (1995, 1996); ratios of methanotroph to chemoautotroph are from Fiala-Médioni *et al.* (2002) and this study.

The small oocyte size and morphology of the prodissoconch II shells of *B. thermophilus* led Lutz *et al.* (1980) to conclude that *B. thermophilus* (found at vents on the East Pacific Rise) has a planktotrophic larval stage with long-distance dispersal capabilities. The similarly small oocyte size (50 μm in diameter) and prodissoconch II size and morphology (shell length = 100 to 600 μm) of *B. azoricus* and *B. heckerae* (this study) led us to a similar conclusion about their larval development strategies. We expected that the post-larval tissues of species with planktotrophic larvae would thus retain a history of a diet of photosynthetically derived organic carbon and nitrogen (*i.e.*, $\delta^{13}C$ of ~ −25.2‰; $\delta^{15}N$ of +8.5 to +9.7‰; Benner *et al.*, 1997). Indeed, diets of photosynthetic origin have been documented for the dispersive stages of vent species. Vent shrimp, for example, have planktonic larvae (Herring and Dixon, 1998), and juveniles of *Mirocaris fortunata* newly arrived at vent sites contain high concentrations of wax ester and (n-3) poly-unsaturated fatty acids, which are presumed to be of photosynthetic origin (Pond *et al.*, 1997). Juveniles of the shrimp species *Rimicaris exoculata* have bulk carbon isotopic compositions that are distinct from adults, but that approach values expected for photosynthetically derived organic material (Polz *et al.*, 1998, Van Dover, 2000).

In this study, however, we found no convincing isotopic evidence for a larval diet of photosynthetically derived organic material in post-larval specimens of either mussel species that we examined. The nitrogen isotopic compositions of post-larvae of both species were so negative (−2.5‰ to −9‰) as to seem difficult, if not impossible, to derive from a diet dominated by photosynthetically derived organic material. The surprising lack of a compelling photosynthetic isotopic signal, together with evidence from transmission electron microscopy that post-larval mussels have well-developed symbiont populations, suggests that the diet of mussel larvae merits further attention. Lipid biomarker analysis of mussel larvae collected in plankton nets or sediment traps away from vent sites, and transmission electron microscopy of gill and mantle tissues in these larvae, should help to resolve the nutritional status and timing of acquisition of endosymbionts in bathymodiolin mussels.

Acknowledgments

We thank the captain and crew of the R/V *Atlantis,* the pilots and technicians of the DSV *Alvin,* and members of the scientific party for their assistance in obtaining biological specimens at sea. We also thank Dr. Joe Scott and Jewel Thomas for their assistance with TEM, and the reviewers of the manuscript for their thoughtful suggestions for improvements. Field collections and laboratory studies were supported by grants to CLVD from the National Science Foundation (Biological Oceanography OCE-988550, OCE-9982999), NOAA's Ocean Exploration Program, the University of North Carolina-Wilmington National Undersea Research Center, and by the College of William and Mary.

Literature Cited

Barry, J. P., K. R. Buck, R. K. Kochevar, D. C. Nelson, Y. Fujiwara, S. K. Goffredi, and J. Hashimoto. 2002. Methane-based symbiosis in a mussel, *Bathymodiolus platifrons,* from cold seeps in Sagami Bay, Japan. *Invertebr. Biol.* **121:** 47–54.

Benner, R., B. Biddanda, B. Black, and M. McCarthy. 1997. Abundance, size distribution, and stable carbon and nitrogen isotopic compositions of marine organic matter isolated by tangential-flow ultrafiltration. *Mar. Chem.* **57:** 243–263.

Berg, C. J., Jr. 1985. Reproductive strategies of mollusks from abyssal hydrothermal vent communities. *Biol. Soc. Wash. Bull.* **6:** 185–197.

Bright, M., and A. Sorgo. 2003. Ultrastructural reinvestigation of the trophosome in adults of *Riftia pachyptila* (Siboglinidae, Annelida). *Invertebr. Biol.* **122:** 345–366.

Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell, and M. E. Lidstrom. 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. *Nature* **325:** 346–348.

Cavanaugh, C. M., C. O. Wirsen, and H. W. Jannasch. 1992. Evidence for methylotrophic symbionts in a hydrothermal vent mussel (Bivalvia: Mytilidae) from the Mid-Atlantic Ridge. *Appl. Environ. Microbiol.* **58:** 3799–3803.

Chaparro, O. R., J. A. Videla, and R. J. Thompson. 2001. Gill morphogenesis in the oyster *Ostrea chilensis. Mar. Biol.* **138:** 199–207.

Charlou, J. L., J. P. Donval, E. Douville, P. Jean-Baptiste, J. Radford-Knoery, Y. Fouquet, A. Dapoigny, and M. Stievenard. 2000. Compared geochemical signatures and the evolution of Menez Gwen (37°50′N) and Lucky Strike (37°17′N) hydrothermal fluids, south of the Azores Triple Junction on the Mid-Atlantic Ridge. *Chem. Geol.* **171:** 49–75.

Charlou, J. L., J. P. Donval, Y. Fouquet, P. Jean-Baptiste, and N. Holm. 2002. Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N, MAR). *Chem. Geol.* **191:** 345–359.

Childress, J. J., and C. R. Fisher. 1992. The biology of hydrothermal vent animals: physiology, biochemistry, and autotrophic symbioses. *Oceanogr. Mar. Biol. Annu. Rev.* **30:** 337–441.

Colaço, A., F. Dehairs, D. Desbruyères, N. Le Bris, and P.-M. Sarradin. 2002. $\delta^{13}C$ signature of hydrothermal mussels is related with the end-member fluid concentrations of H_2S and CH_4 at the Mid-Atlantic Ridge hydrothermal vent fields. *Cah. Biol. Mar.* **43:** 259–263.

Distel, D. L., H. K.-W. Lee, and C. M. Cavanaugh. 1995. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Microbiology* **92:** 9598–9602.

Fiala-Médioni, A., C. Metivier, A. Herry, and M. Le Pennec. 1986. Ultrastructure of the gill of the hydrothermal-vent mytilid *Bathymodiolus* sp. *Mar. Biol.* **92:** 65–72.

Fiala-Médioni, A., Z. P. McKiness, P. Dando, J. Boulegue, A. Mariotti, A. M. Alayse-Danet, J. J. Robinson, and C. M. Cavanaugh. 2002. Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. *Mar. Biol.* **141:** 1035–1043.

Fisher, C. R. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Rev. Aquat. Sci.* **2:** 399–436.

Fisher, C. R. 1993. Oxidation of methane by deep sea mytilids in the Gulf of Mexico. Pp. 606–618 in *Biogeochemistry of Global Change: Radiatively Active Trace Gases,* R.S. Oremland, ed. Chapman and Hall, New York.

Fisher, C. R., J. J. Childress, A. J. Arp, J. M. Brooks, D. Distel, J. A. Favuzzi, H. Felbeck, R. Hessler, K. S. Johnson, M. C. Kennicutt II, S. A. Macko, A. Newton, M. A. Powell, G. N. Somero, and T. Soto. 1988. Microhabitat variation in the hydrothermal vent mussel, *Bathymodiolus thermophilus,* at the Rose Garden vent on the Galapagos Rift. *Deep-Sea Res. A* **35:** 1769–1791.

Fisher, C. R., J. M. Brooks, J. S. Vodenichar, J. M. Zande, J. J. Childress, and R. A. Burke, Jr. 1993. The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. *Mar. Ecol.* **14:** 277–289.

Fujiwara, Y., K. Uematsu, S. Tsuchida, T. Yamamoto, J. Hashimoto, K. Fujikura, Y. Horii, and M. Yuasa. 1998. Nutritional biology of a deep-sea mussel from hydrothermal vents at the Myojin Knoll Caldera. *JAMSTEC J. Deep Sea Res.* **14:** 237–244.

Fujiwara, Y., K. Takai, K. Uematsu, S. Tsuchida, J. C. Hunt, and J. Hashimoto. 2000. Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions. *Mar. Ecol. Prog. Ser.* **208:** 147–155.

Herring, P. J., and D. R. Dixon. 1998. Extensive deep-sea dispersal of postlarval shrimp from a hydrothermal vent. *Deep-Sea Res. Part I* **45:** 2105–2118.

Kochevar, R. E., J. J. Childress, C. R. Fisher, and E. Minnich. 1992. The methane mussel: roles of symbiont and host in the metabolic utilization of methane. *Mar. Biol.* **112:** 389–401.

Le Pennec, M., and P. G. Beninger. 2000. Reproductive characteristics and strategies of reducing-system bivalves. *Comp. Biochem. Physiol. A* **126:** 1–16.

Le Pennec, M., A. Donval, and A. Herry. 1990. Nutritional strategies of the hydrothermal ecosystem bivalves. *Prog. Oceanogr.* **24:** 71–80.

Lutz, R. A., D. Jablonski, D. C. Rhoads, and R. D. Turner. 1980. Larval dispersal of a deep-sea hydrothermal vent bivalve from the Galapagos Rift. *Mar. Biol.* **57:** 127–133.

Nelson, D. C., K. D. Hagen, and D. B. Edwards. 1995. The gill symbiont of the hydrothermal vent mussel *Bathymodiolus thermophilus* is a psychrophilic, chemoautotrophic, sulfur bacterium. *Mar. Biol.* **121:** 487–495.

Page, H. M., A. Fiala-Medioni, C. R. Fisher, and J. J. Childress. 1991. Experimental evidence for filter-feeding by the hydrothermal vent mussel, *Bathymodiolus thermophilus. Deep-Sea Res. A* **38:** 1455–1461.

Paull, C. K., W. Ussler III, W. Borowski, and F. Spiess. 1995. Methane-rich plumes on the Carolina continental rise: associations with gas hydrates. *Geology* **23:** 89–92.

Paull, C. K., R. Matsumoto, P. J. Wallace, N. R. Black, W. S. Borowski, T. S. Collett, J. E. Damuth, G. R. Dickens, P. K. Egeberg, K. Goodman, and others. 1996. Site 996. *Proceedings of the Ocean Drilling Program, Initial Reports* **164:** 241–275.

Paull, C. K., R. Matsumoto, P. J. Wallace, and W. P. Dillon, eds. 2000. *Proceedings of the Ocean Drilling Program,* Scientific Results 164, Ocean Drilling Program, College Station, TX.

Polz, M. F., J. J. Robinson, C. M. Cavanaugh, and C. L. Van Dover.

1998. Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site. *Limnol. Oceanogr.* **43:**1631–1638.

Pond, D. W., D. R. Dixon, and J. R. Sargent. 1997. Wax-ester reserves facilitate dispersal of hydrothermal vent shrimps. *Mar. Ecol. Prog. Ser.* **143:**45–63.

Radford-Knoery, J., J. L. Charlou, J. P. Donval, M. Aballea, Y. Fouquet, and H. Ondreas. 1998. Distribution of dissolved sulfide, methane, and manganese near the seafloor at the Lucky Strike (37°17′N) and Menez Gwen (37°50′N) hydrothermal vent sites on the Mid-Atlantic Ridge. *Deep-Sea Res. I* **45:**367–386.

Raulfs, E. C., S. A. Macko, and C. L. Van Dover. 2004. Tissue and symbiont condition of mussels (*Bathymodiolus thermophilus*) exposed to varying levels of hydrothermal activity. *J. Mar. Biol. Assoc. UK* **84:** 229–234.

Robinson, J. J., M. F. Polz, A. Fiala-Médioni, and C. M. Cavanaugh. 1998. Physiological and immunological evidence for two distinct C_1-utilizing pathways in *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. *Mar. Biol.* **132:**625–633.

Southward, E. C., A. Gebruk, H. Kennedy, A. J. Southward, and P. Chevaldonné. 2001. Different energy sources for three symbiont-dependent bivalve molluscs at the Logatchev hydrothermal site (Mid-Atlantic Ridge). *J. Mar. Biol. Assoc. UK* **81:**655–661.

Streams, M. E., C. R. Fisher, and A. Fiala-Médioni. 1997. Methanotrophic symbiont location and fate of carbon incorporated from methane in a hydrocarbon seep mussel. *Mar. Biol.* **129:** 465–476.

Trask, J., and C. L. Van Dover. 1999. Site-specific and ontogenetic variations in nutrition of mussels (*Bathymodiolus* sp.) from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. *Limnol. Oceanogr.* **44:**334–343.

Van Dover, C. L. 2000. *The Ecology of Deep-Sea Hydrothermal Vents.* Princeton University Press, Princeton.

Van Dover, C. L., D. Desbruyères, M. Segonzac, T. Comtet, L. Saldanha, A. Fiala-Médioni, and C. Langmuir. 1996. Biology of the Lucky Strike hydrothermal field. *Deep-Sea Res.* **43:** 1509–1529.

Van Dover, C. L., P. Aharon, J. M. Bernhard, M. Doerries, W. Flickinger, W. Gilhooly, S. K. Goffredi, K. Knick, S. A. Macko, S. Rapoport, and others. 2003. Blake Ridge methane seeps: characterization of a soft-sediment, chemosynthetically based ecosystem. *Deep-Sea Res. I* **50:**281–300.

Won, Y.-J., S. J. Hallam, G. D. O'Mullan, I. L. Pan, K. R. Buck, and R. C. Vrijenhoek. 2003. Environmental acquisition of thiotrophic endosymbionts in deep-sea mussels of the genus *Bathymodiolus*. *Appl. Environ. Microbiol.* **69:**6785–6792.